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# Haematocyte count and total protein contents in haemolymph of prepual stages *Bombyx mori* CSR2 reared on Probiotics and Artificial dye based diet

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**Abstract**
*Bombyx mori* CSR2 double hybrid were reared on mulberry leaves supplemented with various vital dyes (Neutral red, Rhodamine-B, Acridine orange, Eosin yellow and Light green) on IV and V instars larva. The effect of these particular vital dyes to the *B. mori* in IV and V instar haemolymph were analysed for total haemocyte count (THC), differential haemocyte count (DC) total protein concentration. Impact of vital dyes along with probiotic and supplementation on total count and total protein was analysed. seven groups showed no adverse effect. Maximum THC was observed in group VI supplemented with probiotics and eosin yellow (IV instar – 7157 and V instar – 9052) and similar trend was observed for prohaemocyte (PR), plasmatocyte (PLS) and granulocyte (GRS). For total protein content in heamolymph also group VI registered maximum protein content with 7.57mg/ml in IV instar and 8.87mg/ml in V instar larva. Thus this study clearly indicates that application of vital dyes does not have any negative impact on heamolymph parameters.

**Keywords:** *Bombyx mori*, Vital dyes, THC, Differential Count (DC), Total protein, haemolymph

## Introduction
The silkworm, *Bombyx mori* is the most common monophagous insect domesticated by humans, and the sericulture industry is a major source of income for the world economy (Savithri *et al.*, 2013) [19]. Developmental phases is crucial to understanding silkworm growth as it allows for the determination of the haemolymph's biochemical composition (Zhou *et al.*, 2015 [23]; Shen *et al.*, 2016 [20]; Dong *et al.*, 2017) [7]. There is a relationship between the biochemical concentration fluctuation in the haemolymph in the fifth instar and the onset of spinning (Hirayama *et al.*, 1997 [10], 2000a) [11]. Apart from the haemocyte count and other important chemicals, total protein, total lipids, and α-amylase were crucial for silkworm growth (Rashmi *et al.*, 2013) [17]. Hemocytes are several cell types that circulate throughout the haemolymph (Kerehap *et al*. 2007) such as as prohaemocytes, plasmatocytes, oenocytoids, adipohaemocytes, spherical cells, and granular cells in the majority of insects (Nittono, 1960) [16]. Prohaemocytes are responsible for division, plasmatocyte for phagocytosis, granulocytes, spherulocytes and oenocytoids for storage and secretions (Brehlin and Zachary.,1986). There is inherent variability of haemocytes within a species depending on the development and physiological stages (Beets *et al*, 2008).
Haemocytes are responsible for the immune system's cellular defence mechanism in insects, which uses a range of physical and chemical methods to fight diseases (Siddiqui and Al-Khalifa 2014) [21]. Haemocytes travel freely throughout the haemolymph, the majority of haemocyte is resting on the surface of the many organs that make up the hemocoel (Arumugavel *et al.*, 2021) [2]. Haemocytes in larvae are responsible for the various physiological conditions such as ecdysone hormone production for moulting, oviposition, pupal maturation etc. (Arumugavel *et al.*, 2021) [2]. Present study was carried out to analyse the effect of probiotics and various vital dyes on the haemocytes and total protein count of the prepupal stages of *Bombyx mori*.

## Materials and Methods
### 1. Insect rearing
*Bombyx mori* (Family: Bombycidae) CSR2 double hybrid variety was used in this experiment. In a lab environment, larvae were grown individually at 25±2°C and 65±5% relative humidity. Using a tiny paintbrush, each of the twenty newly hatched larvae were individually transferred to the rearing trays after being selected at random. On fresh mulberry leaves, they were raised from hatching to the fifth instar. Each treatment (n=20), three duplicates were maintained. Beds were regularly cleaned, mature larvae were transferred to the proper cages to spin cocoons (Kebaraj *et al*., 2023) [13].
Mulberry leaves that had been coated with probiotics and vital dyes was given to fourth and fifth-instar larvae, as part of an artificial diet. Apart from moulting stage, the larvae were fed four times a day. More food was given to the larvae as they grew older to suit their nutritional needs.

### 2. Vital dyes based diets
Neutral red, Rhodamine-B, Acridine orange, Eosin yellow and Light green (Nice, chemicals) were some of the common biological vital dyes used in this study. Vital dyes (0.02 b.wt %) was standardized after few trials, a concentration which has no harmful effect on silkworm life cycle (Anumol anto *et al*., 2017) [1]. All the silkworms were kept in a plastic tray with a clean wax paper, and cultured at 30ºC (Kebaraj *et al*., 2023) [13]. Fourth and fifth instars of double hybrid CSR2 Bombyx mori were treated with various vital dye based diets. Group I: Positive control (Probiotics), Group II: Negative control (without probiotics), Group III: Neutral red, Group IV: Rhodomine-B, Group V: Acridine orange, Group VI: Probiotics and Eosin yellow, Group VII: Probiotics and Light green were studied.

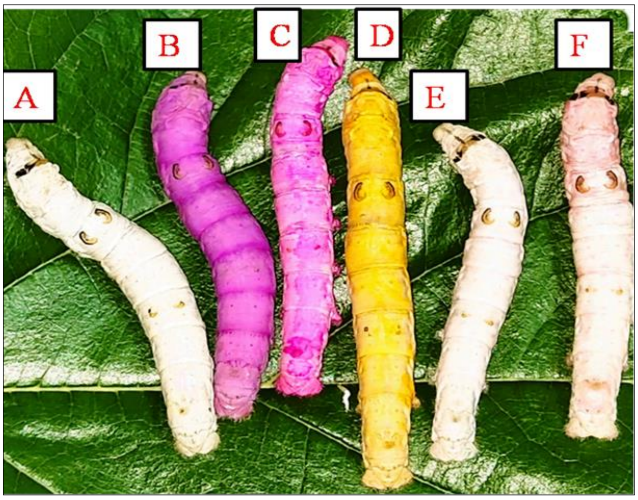


**Fig 1:** CSR2 Double hybrid silkworm Bombyx mori fed with Mulberry leaves fortified with vital dye and probiotics feed) A – Control, B – Neutral Red, C –Rhodamine-B, D – Acridine Orange, E –Light Green, F –Eosin Yellow

### 3. Sample collection

Haemolymph samples were collected by making an incision through one of the prologs and transferred to the eppendorf tubes containing thiourea (0.2M, 4µL) to prevent the sample blackening. The samples were centrifuged for 10 min in 10,000 rpm and upper phase was transferred to new tubes, and stored at -20°C until analysis.

### 4. Total Haemocytes and Differential Haemocyte Count

The haemolymph was transferred onto a glass slide that had first been cleared of impurities using alcohol-soaked cotton and a 20% formalin droplet added. A tiny layer of blood was smeared and positioned within a thin, rectangular glass cover (Arumugavel *et al*., 2021) [2]. Subsequently, the smear was exposed to 25% Giemsa stain, which is prepared with 70% alcohol or methanol, and allowed to air dry for a duration of two minutes. Following that, distilled water was used to carefully clean and dry it. Glass slides was examined under a high power (45X) light microscope. A total of 100 cells were counted for three different cell types: prohaemocytes, plasmatocytes, and granulocytes. Haemolymph were stained by Leishman's stain and observed at 400x.

### 5. Determination of the total protein concentration

Total protein estimation of haemolymph was estimated using Lowry *et al* (1951) [24] method. 1 mL of the haemolymph and 5mL of Copper reagent were mixed thoroughly by vortexing and kept at 38°C for 10 min. 0.5 mL of Folin-Ciocalteau reagent was added to the mixture and incubated at 38°C for 30 min. Bovine serum albumin (BSA) was used as standard. The concentration of the test and standard were measured at 600nm against reagent blank. Using SPSS software version (11.0) the results were analysed.

## Results and Discussion

Haemocytes in haemolymph were responsible for the gaseous exchange as well as buffering mechanism of the body fluids. When compared to the IV instar results, the V instar larval THC and total protein in haemolymph were improved gradually due to the maturation of the instars. Treated group instars showed an increased THC levels as 6747±537 and 8358±467 cells/cu.mm (Group III), 6248±347 and 7085±432 cells/cu.mm (Group V), 5890±524 and 6134±564 cells/cu.mm (Group IV). Among the tested groups, significantly high THC was observed in Group VI: Eosin yellow treated instar as 7157±462 and 9052±490cells/cu.mm for IV and V instars respectively (Table 1). Nahla and Awad (2009), haemocytes in haemolymph perform a variety of vital physiological functions and serve as the primary mediators of the cellular defence response. Silkworm hemopoiesis was shown by an increase in haemocytes in the haemolymph (Russo *et al*., 2001) [18]. During IV and V larval stages there was drastic increase in THC due to increased feed intake (Arunmugavel *et al*., 2021).

Haemocyte cells such as prohaemocyte, plasmatocyte, granulocyte were selected for the analysis due to their vital role in the immunological and moulting process of the instars. Prohaemocyte cells act as the haematopoietic stem cells for the immunological reaction of the haemolymph. Prohaemocyte cells in the Group I: Positive control for IV and V instars were found as 61.4±9.46 and 70.4±6.64 cells/cu.mm respectively. Significantly increased prohameocytes were observed in Group VI: Eosin Yellow as 69.6±8.52 and 78.6±5.02cells/cu.mm for IV and V instars respectively. Among the other vital dyes treatment, increased patterns of prohaemocytes were observed in Neutral red (58.9±9.05 and 68.8±5.77cells/cu.mm), Acridine orange (50.3±8.04 and 61.3±4.99cells/cu.mm) and Rhodomine-B (48.4±7.98 and 57.4±6.54 cells/cu.mm).

Plasmatocyte produces the interstitial fluid which maintains the matrix portion of the haemolymph as well as tissues whereas granulocytes responsible for the production of granular materials which were essential for the coloured granular materials and maturation such as moulting. Plasmatocyte cells in the control IV and V instars were found as 31.4±2.46 and 34.9±3.56cells/cu.mm respectively.

**Table 1:** Total haemocyte count and Differential haemocyte count of *Bombyx mori* CSR2 double hybrid treated with probiotics and various vital dyes

| Experimental Groups | Total haemocyte (Cells/cu.mm) | | Prohaemocyte (Cells/cu.mm) | | Plasmatocyte (Cells/cu.mm) | | Granulocyte (Cells/cu.mm) | |
|---|---|---|---|---|---|---|---|---|
| | IV instar | V instar | IV instar | V instar | IV instar | V instar | IV instar | V instar |
| Group I: Positive control (Probiotics) | 6980±578 | 8694±466 | 61.4±9.46 | 70.4±6.64 | 31.4±2.46 | 34.9±3.56 | 13.7±0.34 | 9.6±0.09 |
| Group II: Negative control without probiotics | 5124±468 | 5908±457 | 42.3±8.99 | 50.4±5.67 | 16.4±3.42 | 22.3±3.46 | 7.1±0.11 | 6.0±0.07 |
| Group III: Neutral red | 6747±537 | 8358±467 | 58.9±9.05 | 68.8±5.77 | 28.9±3.43 | 32.9±2.32 | 11.4±0.13 | 7.93±0.11 |
| Group IV: Rhodamine –B | 5890±524 | 6134±564 | 48.4±7.98 | 57.4±6.54 | 18.4±2.45 | 25.5±1.23 | 7.9±0.12 | 6.44±0.08 |
| Group V: Acridine orange | 6248±347 | 7085±432 | 50.3±8.04 | 61.3±4.99 | 21.5±3.03 | 28.8±2.03 | 8.9±0.15 | 6.81±0.08 |
| Group VI: Eosin yellow | 7157±462 | 9052±490 | 69.6±8.52 | 78.6±5.02 | 36.7±3.43 | 40.3±2.70 | 18.6±0.23 | 13.68±1.03 |
| Group VII: Light green | 4794±324 | 5475±423 | 39.8±6.85 | 43.2±4.78 | 13.1±2.08 | 19.4±1.99 | 6.7±0.14 | 5.4±0.06 |

Significantly (P<0.05) increased plasmatocyte were observed in Group VI: Eosin Yellow as 36.7±3.43 and 40.3±2.70cells/cu.mm for IV and V instars respectively. Among the other vital dyes treatment, increased patterns of plasmatocytes were observed in Neutral red (28.9±3.43 and 32.9±2.32cells/cu.mm), Acridine orange (21.5±3.03 and 28.8±2.03 cells/cu.mm) and Rhodamine-B (18.4±2.45 and 25.5±1.23 cells/cu.mm).

Granulocyte cells in the control for IV and V instars were found as 13.7±0.34 and 9.6±0.09cells/cu.mm respectively. Significantly (P<0.05) increased granulocyte were observed in Group VI: Eosin Yellow as 18.6±0.23 and 13.68±1.03cells/cu.mm for IV and V instars respectively. Among the other vital dyes treatment, increased patterns of granulocytes were observed in Neutral red (11.4±0.13 and 7.93±0.11cells/cu.mm), Acridine orange (8.9±0.15 and 6.81±0.08 cells/cu.mm) and Rhodamine-B (7.9±0.12 and 6.44±0.08cells/cu.mm). Figure 2 showed the blood cells present in Haemolymph stained with Leishman's stain whereas 2A-2F diagram showed the presence of Hyaline cells (HC), Large granulocytes (LGC), Small granulocytes (SGC), Prohaemocyte (PC), and Large granulocytes (LGC) at 400 x magnification.

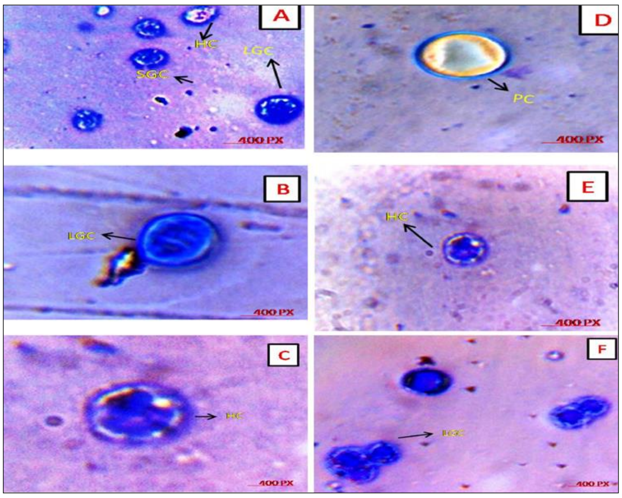


**Fig 2:** Control and treated worms Haemolymph stained with Leishman's

**Fig 2 A**. **Control:** Hyaline cells (HC), Large granulocytes (LGC), Small granulocytes (SGC)
**B. Neutral red:** Large granulocytes (LGC)
**C. Rhodamine – B:** Hyaline cells (HC)
**D. Acridine orange:** Prohaemocyte (PC)
**E. Eosin yellow:** Hyaline cells (HC)
**F. Light green:** Large granulocytes (LGC)

Haemolymph total protein levels in the control were observed as 6.99±0.18 and 8.19±0.22 mg/ml for IV and V instars respectively. When compared to the control and vital dye treated group instar total protein were increased upto 3-fold as 7.57±0.46 and 8.87±0.43mg/ml for IV and V instars of Group VI: Eosin yellow. Vital dyes favoured the growth of the instars which implies on the increased total protein content in the administered group instars. Among the selected two instars – IV and V instars, significantly high haemolymph protein levels were observed in V instar due to the maturation process which promoting cocoon formation.

**Table 2:** Total protein analysis of *Bombyx mori* CSR2 haemolymph treated with probiotics and various vital dye based diets.

| Experimental Groups | Total protein (mg/ml) | |
|---|---|---|
| | IV instar | V instar |
| Group I: Positive control (Probiotics) | 6.99±0.18 | 8.19±0.22 |
| Group II: Negative control without probiotics | 2.78±0.47 | 3.51±0.35 |
| Group III: Neutral red | 6.74±0.48 | 7.65±0.24 |
| Group IV: Rhodamine-B | 3.61±0.48 | 4.70±0.12 |
| Group V: Acridine orange | 5.86±0.21 | 6.85±0.34 |
| Group VI: Eosin yellow | 7.57±0.46 | 8.87±0.43 |
| Group VII: Light green | 2.89±0.46 | 3.27±0.25 |

The total protein content and haemocyte count in the haemolymph of the double hybrid CSR2 *B. mori* treated with probiotics and vital dyes were compared. THC in the instars of the treated group was substantially greater (P<0.05) than that of the instars raised on probiotics (Zhou *et al*., 2008) [22]. When Eri-silkworm larvae were fed an artificial diet, their total protein concentration was almost seven times lower than when they were fed cassava leaves. Haemocytes mediated defence of insect larvae was evident with improved total heamocyte count and differential count. plasmatocytes and prohaemocytes found to be predominant in all larval stages and particularly peaked at IV and V instar larva (Siddique and Kalifa,2014). Granulocyte percentage was more during final instar and the role of granulocyte was to attract plasomatocyte (Riberio and Brehlin, 2006).

Total protein content in haemolymph was observed in IV and V instar larva showed increasing trend as it is essential for spinning and cocoon formation. In conclusion it appears that probiotic supplementation along with vital dyes doesn't have any negative impact. Whereas it shows normal haemocyte composition to meet the immune response and protein concentration for the need of spinning and Cocoon formation.

## Conclusion

*B. mori* fed with CSR2 double hybrid mulberry leaves on IV and V instars based on the artificial diet between the groups were studied. Probiotics and different vital dyes namely Neutral red, Rhodamine-B, Acridine orange, Eosin yellow and Light green based diet ingested *B. mori* CSR2 IV and V instars showed an improved haematocyte and total protein levels in haemolymph. Among the dyes, Eosin yellow dye with probiotics supplemented showed a significant result collectively. While comparing with control instars, vital dyes administration through diet enhanced the cell count and protein content of the haemolymph. This study concluded that vital dye along with probiotics applied to the mulberry leaves ingested by *B. mori* CSR2 has no adverse effect on their growth and physiological mechanism.

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